CpG-A and CpG-B oligonucleotides differentially enhance human peptide–specific primary and memory CD8⁺ T-cell responses in vitro

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Two distinct types of CpG oligodeoxynucleotide (ODN) have been identified that differ in their capacity to stimulate antigen-presenting cells: CpG-A induces high amounts of interferon-α (IFN-α) and IFN-β in plasmacytoid dendritic cells (PDCs), whereas CpG-B induces PDC maturation and is a potent activator of B cells but stimulates only small amounts of IFN-α and IFN-β. Here we examined the ability of these CpG ODNs to enhance peptide-specific CD8⁺ T-cell responses in human peripheral blood mononuclear cells (PBMCs). The frequency of influenza matrix–specific “memory” CD8⁺ T cells was increased by both types of CpG ODN, whereas the frequency of Melan-A specific “naive” CD8⁺ T cells increased on stimulation with CpG-B but not with CpG-A. The presence of PDCs in PBMCs was required for this CpG ODN-mediated effect. The expanded cells were cytotoxic and produced IFN-γ on peptide restimulation. Soluble factors induced by CpG-A but not CpG-B increased the granzyme-B content and cytotoxicity of established CD8⁺ T-cell clones, each of which was IFN-α/β dependent. In conclusion, CpG-B seems to be superior for priming CD8⁺ T-cell responses, and CpG-A selectively enhances memory CD8⁺ T-cell responses and induces cytotoxicity. These results demonstrate distinct functional properties of CpG-A and CpG-B with regard to CD8 T cells. (Blood. 2004;103:2162-2169) © 2004 by The American Society of Hematology

Introduction

The induction of cytotoxic CD8⁺ T cells (CTLs) in the absence of antigen-carrying living pathogens (viruses or intracellular bacteria) is a major goal of new vaccine strategies directed against established tumors and a range of infectious diseases. Since Freund's first report on mycobacterial extracts, the use of vaccine adjuvants has become a widespread but incompletely understood practice to promote specific T- and B-cell responses. In recent years it has become clear that conserved microbial molecules are recognized by the innate immune system by Toll-like receptors, a new family of pattern recognition receptors eliciting specific signaling cascades that ultimately result in enhancing and guiding T- and B-cell responses (for reviews, see Akira et al. and Bendelac et al).

Recognition of bacterial DNA by Toll-like receptor 9 (TLR9) is based on the presence of unmethylated CG dinucleotides in particular sequence contexts (Cpg motifs). Synthetic oligodeoxynucleotides (ODNs) containing such Cpg motifs (Cpg ODNs) mimic bacterial DNA and induce a coordinated set of immune responses that comprise innate immunity and acquired T1,1-β-like cellular and humoral immunity (for a review, see Krieg). In mice and in nonhuman primates, CpG ODNs boost the efficacy of vaccines against bacterial, viral, and parasitic pathogens. Several reports also demonstrate the potential of CpG ODNs to enhance CTL responses in mice. However, thus far this has not been shown for humans. Because of evolutionary divergence, optimal CpG motifs differ between mice and humans. In mice but not in humans, cells of the myeloid lineage, such as monocytes and myeloid dendritic cells, directly respond to CpG ODN, leading to differences in the target cells activated and the cytokines induced by CpG ODN and limiting the extrapolation of results from mice to primates and humans.

Recently, 2 types of CpG ODN have been identified based on distinct immunologic activities on human plasmacytoid dendritic cells and B cells. CpG-A (eg, ODN 2216 and ODN 1585) are characterized by poly G tails with phosphorothioate linkages flanking a central palindromic CpG motif-containing sequence with a phosphodiester backbone. CpG-A induces high amounts of interferon-α/β (IFN-α/β) in plasmacytoid dendritic cells (PDCs) (5 pg/PDC) but is relatively weak at activating B cells. CpG-B (eg, ODN 2006), which has a phosphorothioate backbone and lacks poly-G tails, strongly promotes the maturation and activation of PDCs but induces only small amounts of IFN-α/β. Unlike CpG-A, CpG-B is weak at activating natural killer (NK) cells but strongly stimulates B cells. Similar types of CpG ODNs were described by Klimman et al. based on NK cell activation and IFN-γ production in peripheral blood mononuclear cells (PBMCs) (their D and K type CpG ODNs correspond to A and B type ODNs, respectively).
Recent results suggest that PDCs and B cells are the only cells within human PBMCs that are directly responsive to CpG ODN. Both NK cell activation and IFN-γ induction seem to be indirect effects of CpG ODN mediated through PDCs.14,21

In the present study we examined the activity of these 2 types of CpG ODN to enhance the induction of peptide-specific CTLs. Our studies demonstrate for the first time that CpG ODNs are capable of promoting peptide-specific CD8+ T-cell responses in the human immune system. The results provide evidence that CpG-A and CpG-B ODNs differ in their ability to prime naive or to boost memory CD8+ T cells and to confer cytotoxic activity.

Materials and methods

Oligodeoxynucleotides and synthetic peptides

Completely and partially phosphorothioate-modified ODNs were provided by the Coley Pharmaceutical Group (Wellesley, MA) (small letters indicate phosphorothioate linkage; capital letters, phosphodiester linkage) and were stored at –80°C. ODNs used in this study were synthesized on a multiple peptide synthesizer (peptide synthesizer 433A; Applied Biosystems, Foster City, CA) by the core facility of the German Cancer Research Center, Heidelberg, Germany. Oligodeoxynucleotides used were the following: ODN 2006, 5'-CCGAAGCGTTCgggggG-3'; ODN 2243 the GC control to ODN 2216, 5'-TGACGATGCgggggG-3'; ODN 1585, 5'-ggGTTCAAGTTTGGgggggG-3'; ODN 2216, 5'-ggGGGAGCACATGCTGgggggG-3'. The HLA-A*0201-restricted peptides derived from the melanoma-associated differentiation antigen Melan-A/MART-1 (A26-35 A27L), from the influenza matrix protein GILGFVFTL (referred to as Flu matrix x35.66) and from the HIV pol protein ILKPEVHG (referred to as HIV pol x176-195), were synthesized on a multiple peptide synthesizer (peptide synthesizer 433A; Applied Biosystems, Foster City, CA) by the core facility at the LSFA Research Institute Munich (Dr Arnold). All peptides were more than 90% pure, as indicated by high-performance liquid chromatography (HPLC) analysis. Lyophilized peptides were diluted in 30% dimethyl sulfoxide (DMSO) and were stored at –20°C. Pyrogen-free reagents were used for all dilutions. CpG ODN and peptides were found to be negative for endotoxin using the LAL assay (BioWhittaker, Walkersville, MD; lower detection limit, 0.1 EU/mL).

Preparation, culture, and expansion of peptide-specific CD8+ T cells

Human PBMCs were isolated from buffy coats (provided by the Institute of Immunology and Transfusion Medicine, University of Greifswald, Germany) or freshly drawn peripheral blood by Ficol-Hypaque (Biochrom, Berlin, Germany) density gradient centrifugation. Blood donors were 18- to 68-year-old healthy men and women who were negative for HIV, hepatitis B virus (HBV), and HCV infection. Informed consent was obtained from all donors. For this study HLA-A2-positive donors were selected after PBMCs were stained with an HLA-A2-specific antibody (BB7.2; hybridoma from ATCC) and analysis by flow cytometry. To increase the precursor frequency of peptide-specific cells, CD8+ T cells were enriched by one round of positive selection using anti-CD8 antibody beads and MACS-technology (Miltenyi Biotec) according to the manufacturer’s protocol (less than 0.02% PDCs identified as Lin−, CD123–, HLA-DR− and less than 0.1% B cells after depletion). Medium was changed every second day after day 5, but no further ODN or peptides were added. After 10 to 14 days, the cells were harvested and analyzed as indicated.

Flow cytometric immunofluorescence analysis

Monoclonal antibodies against human CD3FITC (clone UCHT1), CD8PerCP (clone RPA-T8), CD8αFITC (clone CD28.2), CD56PerCP (clone B159), CD123PE (clone 7G3), HLA DRPerCP (clone L243), Foxp3ABFITC (clone NOK1), and IFN-γPE (clone B27) were purchased from PharMingen/ Becton Dickinson (Heidelberg, Germany). The anti-human SLAMFITC antibody (clone A12) was kindly provided by Lewis Lanier (University of California, San Francisco), and the anti-granzyme-BPE antibody was from Hölzel Diagnostika (Cologne, Germany). Phycoerythrin (PE)-coupled HLA-A2/Melan-A26-35 A27L, HLA-A2/Flu matrix x35.66, and HLA-A2/HIV pol x475-484 tetramers (kindly provided by Philippe Guillaume, Ludwig Institute for Cancer Research, Lausanne branch, Epalinges, Switzerland) were synthesized as previously described.24 For surface analysis, cells were harvested and stained in 50 µL phosphate-buffered saline (PBS)/2% human serum albumin (HSA) with the indicated tetramers for 40 minutes at 20°C, and then the fluorescence-labeled antibodies were added. After another 20 minutes of incubation on ice, the cells were washed once and analyzed on a FACSCalibur (Becton Dickinson). In 3-color stainings, TO-PRO-3 iodide (Molecular Probes, Eugene, OR), a DNA intercalating dye with fluorescence characteristics similar to that of allophycocyanin (APC), was added immediately before analysis to exclude dead cells. To assess antigen-specific IFN-γ production, cells were harvested after 10 to 14 days, washed once, and restimulated in 96-well, round-bottom culture plates in 200 µL medium with 10 µM cognate peptide or the HIV pol x176-195 peptide as a control. After 2-hour incubation at 37°C, 1 µg/mL brefeldin A (Sigma, Munich, Germany) was added. After 4-hour incubation at 37°C, cells were harvested, and intracellular cytokine staining was performed as previously described.18 Data were analyzed using CellQuest software (Becton Dickinson).

Generation of peptide-specific CD8+ T-cell clones

Melan A26-35 A27L and Flu matrix x35.66 peptide-specific CD8+ T-cell clones were generated from PBMCs of HLA-A2-positive healthy volunteers. PBMCs were stimulated in vitro with Melan A26-35 A27L and Flu matrix x35.66 peptides as described.18 After 14 days, Melan A26-35 A27L and Flu matrix x35.66 peptide-specific CD8+ T cells were labeled with HLA-A2/Melan A26-35 A27L tetramers and HLA-A2/Flu matrix x35.66 tetramers, respectively, and anti-CD8 antibodies, subsequently sorted directly into 96-well plates using a FACStarPLUS flow cytometer (Becton Dickinson, Heidelberg, Germany) at a frequency of 1 cell per well and expanded as previously described.25 All T-cell clones were grown in IMDM supplemented with 8% human AB serum, 100 IU/mL IL-2, 0.25 µg/mL phytohemagglutinin (PHA; Sigma, Munich, Germany), and irradiated feeder cells (3 × 109 allogeneic PBMCs plus 1.5 × 105 721 LCL cells per well). At 2 to 4-week intervals, the clones were passaged with feeder cells and PHA. The phenotype and the expression of an HLA-A2/Melan A26-35 A27L- and an HLA-A2/Flu matrix x35.66-specific T-cell receptor (TCR), respectively, were confirmed by flow cytometry for each clone used in subsequent experiments. For the activation assays, clones were washed, incubated for 18 hours in the cell-free supernatant derived from CpG ODN-stimulated PBMCs (2 × 106/mL; 6 µg CpG ODN for 24 hours; no peptide added), washed again, and then used as effectors in chromium Cr 51 release assays. In some experiments blocking antibodies to interferon type 1 (a combination of polyclonal rabbit anti-IFN-α (5000 neutralizing U/mL) and rabbit anti-IFN-β (2000 neutralizing U/mL) antibodies, together with 20 µg/mL monomeric mouse antihuman IFN-α/β receptor chain 2 antibody (all from PBL, New Brunswick, NJ) or IL-12 (clone C8.6, 3 µg/mL; BD/PharMingen), were added at the beginning of the 18-hour incubation time of the T-cell clones in CpG-induced supernatants.
Assay of in vitro cytolytic activity

Antigen-specific lytic activity was measured by performing a standard 51Cr release assay using peptide-pulsed HLA-A2–positive, TAP-negative T2 cells (lymphoblast cell line, ATCC CRL-1992) as target cells. Briefly, 2 to 5 × 10^6 T2 cells were pulsed in 150 µL medium with 10 µM cognate or control peptide for 2 hours at 37°C and subsequently were labeled with 100 µCi (3.7 MBq) 51Cr (NEN Life Science Products, Köln, Germany) for another hour at 37°C. Cells were washed 4 times and were used as target cells (3 × 10^5 cells/well) for effector T cells (E/T ratios as indicated) in 96-well, round-bottom plates. After 4-hour incubation at 37°C, 50 µL supernatant/well were harvested, and radioactivity was measured on a gamma counter. Maximum release was assessed by the addition of Triton X–100 (0.75% of the CD8 T-cell responses in unseparated PBMCs. The frequency of antigen-specific CD8+ T cells in PBMCs from HLA-A2–positive donors was stimulated with the Flu matrix58-66 peptide or a control peptide. In agreement with the GC control ODN to ODN 2216 (ODN 2243) showed no increase in the frequency of tetramer+ T cells (Figure 1C).

In addition to tetramer staining, we measured the number of IFN-γ-producing cells on Flu matrix58-66 peptide restimulation (Figure 1D–E). Similar to the results obtained by tetramer staining, both types of ODN induced the frequency of Flu matrix58-66 peptide–specific IFN-γ-producing cells (from 1.6% IFN-γ+ cells of all CD8+ T cells without ODN to 4.4% with ODN 2006, 3.1% with ODN 1585, 2.9% with ODN 2216; n = 13). Stimulation with a control peptide (HIV p17-40 L) showed less than 0.2% IFN-γ-positive CD8+ T cells. The number of peptide-specific CD8+ T cells detected by intracellular IFN-γ staining on peptide restimulation correlated with tetramer staining, but on a lower level. On average, approximately one third of tetramer-positive cells produced IFN-γ in response to peptide restimulation. There was a trend for ODN 2006 to induce higher numbers of peptide-specific IFN-γ–producing cells compared with the other ODNs, but this difference was not statistically significant. (P = .27 for differences between ODN 2006 and ODN 1585; P = .34 for differences between ODN 2006 and ODN 2216.) For some donors we assessed the cytotoxic capacity of CD8+ T cells within PBMCs against the HLA-A2–positive T2 cell line pulsed with the Flu matrix58-66 peptide or a control peptide. In agreement with the higher number of peptide-specific CD8+ T cells, the Flu matrix58-66 peptide–specific cytotoxicity of PBMCs was enhanced in the presence of CpG-A and CpG-B but not in the presence of a control ODN to 2006 (ODN 2137) (Figure 1F). The percentage specific lysis ± SEM increased from 17.8% ± 3.5% without CpG ODN to 44% ± 8.8% (ODN 2006) and 54% ± 10.1% (ODN 2216) in the presence of CpG-ODN (E/T ratio 27:1; n = 4; P < .05 for differences between medium and CpG ODN-stimulated conditions). Together these results demonstrate that both CpG-A and CpG-B increase the frequency of influenza peptide–specific CD8+ T cells and that these T cells are functionally active in terms of IFN-γ production on peptide restimulation and peptide-specific cytolytic activity.

CpG-B but not CpG-A supports priming of naive Melan-A–specific CD8+ T cells toward IFN-γ–producing cytotoxic CTLs

As a model for priming of naive CD8+ T cells, we tested the ability of different CpG ODNs to enhance the induction of Melan A26-35
A27L–specific naïve CD8+ T cells (Figure 2A–B), and this effect was CpG dependent (Figure 2C). In contrast, CpG-A (ODN 1585; ODN 2216) was unable to enhance the frequency of Melan A26–35 A27L–specific CD8+ T cells (from 1.1% HLA-A2/Melan A26–35 A27L tetramer+ cells of all CD8+ T cells without ODN to 1.4% with ODN 1585 and 1.1% with ODN 2216; n = 16). Consistent with the number of tetramer+ cells, IFN-γ+ CD8+ T cells increased from 0.5% without CpG ODN to 1.5% with ODN 2006, 0.5% with ODN 1585, and 0.4% with ODN 2216; n = 12; Figure 2D). Stimulation with a control peptide (HIV pol476–484) showed less than 0.2% IFN-γ+ positive CD8+ T cells. In agreement with the higher numbers of Melan-A–specific CD8+ T cells with CpG ODN 2006 in PBMCs, cytotoxicity toward peptide-loaded target cells was also increased (Figure 2E). The percentage specific lysis ± SEM increased from 38.7% ± 15.6% without CpG ODN to 66.7% ± 21.2% in the presence of ODN 2006 (E/T ratio 20:1; n = 3; P = .16). When we used HLA-A2–restricted peptides derived from the HIV pol or gag proteins as model antigens for a primary immune response in HIV-negative donors, unlike for Melan-A, we could not detect peptide-specific CD8+ T cells within
PBMCs (neither by tetramer-staining nor by IFN-γ production; data not in figure). The frequency of naïve T cells specific for these HIV antigens seemed to be too low to allow efficient priming and expansion in our vitro system.

**PDCs are required for the CpG ODN-induced enhancement of peptide-specific CD8+ T-cell responses**

Next we studied whether PDCs and B cells, the only TLR9-expressing cell subsets in PBMCs, contribute to the expansion of peptide-specific CD8+ T cells in response to CpG ODN. PBMCs were depleted of PDCs and B cells before stimulation with peptide and CpG ODN. In a first set of experiments, PBMCs depleted of PDCs or of B cells were stimulated with Flu matrix58-66 peptide in the presence or absence of CpG-A (Figure 3). In the absence of CpG-A, the depletion of PDCs or B cells did not significantly change the frequency of peptide-specific T cells (frequency of Flu-peptide–specific T cells: PBMCs, 13% ± 7.5%; PBMCs depleted of B cells, 14% ± 4.8%; PBMCs depleted of PDCs, 12% ± 8.4%; P > .6 depleting vs undepleted; not in figure). In contrast, depletion of PDCs abrogated the CpG-A-induced increase of Flu Matrix58-66–specific T cells. The depletion of B cells had no significant effect on the frequency of Flu matrix58-66–specific T cells (Figure 3, left panel). In contrast, the depletion of B cells enhanced the frequency of Melan-A–specific T cells (Figure 3, right panel). However, the depletion of B cells and PDCs completely abrogated the CpG-mediated effect (Figure 3, right panel). Again, in the absence of CpG, the depletion of PDCs or B cells did not significantly change the frequency of peptide-specific T cells (frequency of Melan-A–specific T cells: PBMCs, 0.18% ± 0.11%; PBMCs depleted of B cells, 0.27% ± 0.21%; PBMCs depleted of B cells and PDCs, 0.15% ± 1.2%; P > .1 depleting vs undepleted; not in figure). Together these data indicated that the presence of PDCs in PBMCs is required for the CpG-mediated enhancement of Flu- and Melan-A peptide–specific T cells.

**CD8+ T cells generated in the presence of CpG ODN express a phenotype associated with cytotoxicity and terminal differentiation**

An effective CD8+ T-cell response depends on the quantity and the quality of the T cells generated. Antigen-specific CD8+ T cells expanded in vitro or in vivo may lack effector functions and cytotoxicity. To monitor the functional activity of CD8+ T cells in vaccination studies with tumor antigen–derived peptides, the down-regulation of CD28 and CD45RA and the up-regulation of signaling lymphocytic activation molecule (SLAM) have been described to correlate with the differentiation toward effector cells. The expression of CD56 is reported to correlate with lytic activity of ex vivo–analyzed CD8+ T cells.

To examine whether CpG ODNs not only increase the frequency of peptide-specific CD8+ T cells but also affect their phenotype, we measured the expression of CD28, CD56, and SLAM on Flu matrix58-66 peptide–specific cells expanded in the absence or presence of different CpG ODNs. Compared with nonantigen-specific CD8+ T cells, Flu matrix58-66 tetramer+ cells showed a lower expression of CD45RA (not in figure) and CD28 and a higher expression of SLAM (P < .05 comparing the expression on antigen-specific and antigen-nonspecific T cells for each condition) (Figure 4A-B). Thus, antigen-specific T cells carried a phenotype compatible with that of terminally differentiated effector cells. No difference was found regardless of whether peptide-specific T cells were generated in the presence or absence of CpG ODN, and there was no significant change in the expression of these markers between the different types of CpG ODNs. The situation was different for CD56, which is reported to be associated with cytolytic activity. Although there was no significant difference in CD56 expression between antigen-specific and antigen-nonspecific CD8+ T cells without CpG ODN (P = .29), the presence of CpG ODNs during CD8+ T-cell expansion led to increased CD56 expression on peptide-specific CD8+ T cells (P < .01 for differences between no ODN and any of the CpG ODNs; Figure 4B). Within the 2 types of CpG ODNs used, CpG-A (CpG ODN 2216 and ODN 1585) was more potent than CpG-B (ODN 2006) to up-regulate CD56 expression (P < .01 for differences between ODN 2006 and ODN 2216, and P = .05 for differences between ODN 2006 and ODN 1585). Experiments with GC control ODN to ODN 2006 and ODN 2216 demonstrated the CpG specificity of that effect (Figure 4C). Similar changes were seen on Melan-A–specific CD8+ T cells (data not shown).

**CpG-A ODN–induced IFN-α/β increases the lytic activity and the granzyme-B content of pre-established CD8+ T-cell clones**

The increased CD56 expression on Flu matrix58-66–specific CD8+ T cells in response to CpG ODN suggested that CpG not only increases the frequency of peptide-specific CD8+ T cells but also enhances their cytotoxic activity. To test the effect of CpG ODN on the cytotoxic activity of T cells on a per cell basis, we generated Melan A26-35 A27L–specific CD8+ T-cell clones. Established T-cell clones were incubated in the presence of supernatants derived from PBMCs stimulated with ODN 2006, ODN 2216, or ODN 2243 or without ODN. After 18 hours, the cytotoxic activity of T-cell clones against Melan A26-35 A27L–pulsed T2 cells was determined. As seen in Figure 5A-B, supernatant derived from CpG-A (ODN 2216)–, but not from CpG-B (ODN 2006)–, stimulated PBMCs increased the lytic activity of the T-cell clones (P = .001). This effect was CpG-specific, as demonstrated by the non-CpG control ODN 2243 (Figure 5B-C). Increased cytotoxic activity of T-cell clones in response to CpG-A–derived supernatant correlated with the higher induction of CD56 expression (compare Figure 4B) by CpG-A compared with CpG-B ODN. Unlike CpG ODN–induced PBMC supernatant,neither CpG-A nor CpG-B was able to directly activate T-cell clones (data not shown). These results indicate that soluble factors induced by CpG-A confer increased cytotoxic activity to peptide-specific CD8+ T cells and that the direct cell-to-cell contact between T cells and APC within PBMCs is not required for this activity.
Cytotoxic T cells kill their target cells mainly by 2 different mechanisms, the Fas/Fas-ligand pathway and through the effects of perforin and granzymes. To further characterize the mechanisms that lead to enhanced T-cell lytic activity, we measured the intracellular granzyme-B content and the expression of Fas-ligand on T-cell clones after incubation in ODN 2216-induced supernatant by flow cytometry. Compared with the isotype control, no expression of Fas-ligand could be detected on the clones even if they were not incubated with CpG-conditioned supernatant. However, increased cytolytic activity of the T-cell clones was associated with an increase in intracellular granzyme-B content (Figure 5D).

Because IFN-α is described to increase the cytotoxicity of activated CD8⁺ T cells and NK cells, we further examined the role of IFN-α/β in the ODN 2216-induced supernatants for the increased cytotoxicity of peptide-specific clones. Indeed blocking interferon type 1 during the incubation time in supernatants derived from PBMCs stimulated with ODN 2216 reversed the cytotoxicity and increase of granzyme-B content almost to the level of the control without CpG ODN (Figure 5C-D). Blocking antibodies to IL-12 led only to a small reduction in the cytotoxicity (not in figure) and of granzyme-B content (Figure 5D). Blocking antibodies to IFN-γ had no effect (data not shown).

**Discussion**

Progress in the immunotherapy of cancer and of infections with intracellular pathogens and viruses depends on vaccine strategies that induce a large number of antigen-specific CD8⁺ T cells with high cytotoxic activity. Today vaccines that meet these criteria are mainly based on viable pathogens. Activation of the innate immune system primarily based on viable pathogens. Activation of the innate immune system...
system by conserved microbial products triggers a cascade of events that have profound effects on adaptive immunity. The present study provides evidence that CpG ODNs as molecular mimics of bacterial DNA may serve as vaccine adjuvants for the generation of peptide-specific CTL in humans even in the absence of a live vaccine.

We demonstrate that CpG ODN improves the generation and cytotoxic activity of peptide-specific human CD8+ T cells within PBMCs in vitro. Interestingly, 2 types of CpG ODN, CpG-A and CpG-B, showed distinct functional profiles depending on the type of CD8+ T cells exposed to CpG ODN. Although CpG-B (ODN 2006) enhanced the frequency of Melan-A– and influenza matrix–specific CD8+ T cells, CpG-A (ODN 2216, ODN 1585) only expanded influenza matrix–specific CD8+ T cells. Within PBMCs both types of CpG ODN conferred peptide-specific cytotoxicity and IFN-γ production to T cells; however, only CpG-A induced a CD8+ T-cell phenotype associated with increased lytic activity. Quantitative analysis of cytotoxicity in peptide-specific T-cell clones revealed that CpG-A was more active than CpG-B in supporting cytotoxic activity and granzyme-B content on a per cell basis in CD8+ T cells and that this was mediated mainly by IFN-α/β.

The distinct response of Melan-A– and influenza matrix–specific CD8+ T cells could be attributed to the distinct differentiation stages of T cells. Evidence in the literature shows that Melan-A–specific CD8+ T cells carry a naive phenotype, whereas influenza matrix–specific CD8+ T cells carry a memory phenotype. The concept of differentiation dependence of the effect of CpG-A compared with CpG-B is supported by our finding that CpG-A failed to activate naive Melan-A–specific T cells but induced the cytotoxic activity of established Melan-A–specific T-cell clones even though the same antigenic peptide was used. However, it cannot be excluded that different molecular characteristics of the 2 antigenic peptides also contribute to the observed differences.

Neither CpG-A nor CpG-B activates CD8+ T cells directly. Previous studies identified PDCs and B cells as the only 2 cell types within primary human PBMCs that express TLR9 and are sensitive to CpG ODN. The differences between CpG-A and CpG-B with regard to CD8+ T-cell expansion must, therefore, be mediated by PDCs and B cells. Depletion of PDCs revealed that the presence of PDCs within PBMC was not necessary to mediate the effects of CpG-A and CpG-B. Although the presence of PDCs was essential, the expansion of CD8+ T cells was higher within PBMCs than with isolated PDCs as antigen-presenting cells (V.H., unpublished results, 2003), suggesting that other cell subsets contribute to CD8+ T-cell expansion. In contrast to PDCs, the depletion of B cells did not decrease the expansion of influenza-reactive T cells, and it even increased the expansion of Melan-A–specific T cells. It is interesting that though dendritic cells can activate naive and memory T cells, B cells have been reported to activate memory T cells but to render naive T cells tolerant. In our in vitro model, peptide presentation takes place simultaneously on DCs and B cells, which could explain the enhanced CTL response of naive Melan-A–specific cells, when the B cells were depleted. Similar increases in CTL responses after B-cell depletion have been described in tumor and transplantation models in mice.

Besides cell contact–dependent mechanisms between T cells and antigen-presenting cells, CpG-induced cytokines impact on T-cell function. CpG-A is known to stimulate PDCs to release large amounts of IFN-α. IFN-α activates NK cells and induces partial activation of memory CD8+ T cells and proliferation, IFN-γ production and lytic activity of γδ T cells known to carry a memory phenotype. This is consistent with the role of IFN-α to promote preactivated or memory T cells, an effect which is partly mediated via the induction of IL-15 in myeloid cells. Interestingly, CpG-A (also called D-type) through IFN-α has been reported to induce monocyte-derived dendritic cells that produce IL-15. In addition to memory T-cell expansion, IFN-α has been shown to increase the cytotoxicity of activated CD8+ T cells and NK cells. Together, the present study and the previous results suggest that CpG-A recruits innate effector cells (NK cells and γδ T cells), selectively expands memory CD8+ T cells, and confers cytotoxicity through IFN-α.

Unlike the effects of IFN-α on memory T cells, IFN-α is known to have a strong antiproliferative effect on naive T cells and to inhibit the progress from G1 to the S phase of the cell cycle after T-cell receptor stimulation and to inhibit IL-12 production in human monocytes and myeloid DCs. The antiproliferative effect of IFN-α known from the literature is in agreement with our observation that priming of CD8+ T cells carrying a naive phenotype (Melan-A–specific CD8+ T cells) was not supported by CpG-A (inducing high amounts of IFN-α in PDCs). However, priming of CD8+ T cells was mediated by CpG-B, which is weak at inducing IFN-α in PDCs. In previous studies, we found that CpG-B in combination with CD40L is a potent stimulus for the production of IL-12 in PDCs and that it promotes PDC-dependent priming and differentiation of naive CD4+ T cells toward T helper type 1. Results from the present study now indicate that CpG-B also supports the priming of functionally active antigen-specific CD8+ T cells.

In conclusion, our results suggest that CpG-B may be the superior vaccine adjuvant when the induction of a primary CTL immune response is needed, such as in prophylactic vaccines. On the other hand, CpG-A may be superior to expand preexisting T cells and to regain T-cell responsiveness and cytotoxicity. However, the activity of CpG ODN in vivo, especially in patients with cancer, cannot be predicted based on in vitro data. In vivo, in a recent study Verthelyi et al compared 2 types of CpG ODN (K-type ODN, similar to CpG-B; D-type ODN, similar to CpG-A) as adjuvants for a heat-killed leishmania vaccine in rhesus macaques. In agreement with our results, K-type ODN (CpG-B) induced more antigen-specific IFN-γ–producing T cells than D-type ODN (CpG-A) concerning a primary immune response. Ongoing clinical trials will provide further insight into the activity of CpG ODNs as immune adjuvants for peptide vaccines against cancer.

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References


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